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SOME ASPECTS OF ENZYME TECHNOLOGY

by
Pat M. Lee

ABSTRACT

The major concern of biotechnology is to produce products via biotransformation. Biotechnology has been generally accepted as the new technology in the 21st century which will bring a great impact to our everyday life. Enzyme technology is one of the important areas in biotechnology. Our research activities in biotechnology include the following three important aspects.

(i) Utilization of readily available, low cost, and non-toxic alginate to immobilize aminoacylase for the production of *L*-phenylalanine under mild conditions.

Calcium alginate beads were stabilized by coating with stabilizing agents.Several systems have been studied in detail. Among these systems investigated, PLL-coated calcium alginate beads was found to be the best. It possessed great enzyme stability and operational stability and may be used for industrial production of *L*-phenylalanine. This work is presented in this paper.

(ii) Employing proteolytic enzymes, elastase and thermolysin in non-conventional solvent systems for the coupling of *N*-benzyloxycarbonyl-Aspartic acid and phenylalaninemethyl ester to produce the precursor of dipeptide sweetener, aspartame.

The reaction conditions for the synthesis of the aspartame precursor were investigated in details. The optimal conditions for this synthesis in the non-conventional solvent systems has been established.

(iii) Isolation and characterization of enzymes from Malaysia cocoa beans.

Polyphenoloxidase and proteases have been isolated and characterized. Studies were also conducted to delineate the enzyme involvement in the flavour production of cocoa beans.

Keywords: Calcium alginate (CAG), Poly-*L*-lysine (PLL), Enzyme Technology, biotechnology, aminoacylase, L-phenylalanine.

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1 INTRODUCTION

1.1 Enzymes

Enzymes are the cornerstone of life. They are biocatalysts which control and regulates all of the physiological activities of synthesis, decomposition and partial conversion of biological molecules in the living systems. They increase the rates of reactions without themselves being modified. The word enzyme is derived from the Greek meaning "in yeast" and was first coined by Kuhne (1975) in 1878. It was recognised that enzyme is a protein by the beginning of 20th century. The enzyme molecules are built from different amino acids in a sequence specific manner. The first protein sequence of ribonuclease was determined in 1960. Enzyme molecules also possess a unique 3-dimensional structure which possess a catalytic center, one or more substrate binding sites. The strategic 3-dimensional arrangement of the amino acid residues in the catalytic center and the cooperative action of these residues give rise to the catalytic power of enzyme. In 1965, the first three dimensional structure of enzyme, lysozyme, was determined by X-rays crystallography (Blundell and Johnson, 1976).

Enzymes were being used as processing aids for many years before their essential nature was understood. In fact, production of enzyme for industrial use is an ancient art. and probably the coagulation of milk to produce cheese with the calf stomach extract was the first artificial use of enzymes for thousands of years. It was only realised recently that the coagulation was caused by the proteolytic enzyme, renin, present in the calf's stomach.

Enzymes exhibit three important characteristics:

(i) It has high catalytic power.
It can accelerate rates of reactions by factors of 10^8 to 10^{14} or higher whereas nonenzymatic catalytic reactions are usually 10^2 to 10^6 time faster than uncatalysed reactions.

(ii) It has specificity.
Most enzymes are highly specific both in the nature of substrates which they catalyse and also in the reaction they catalyse. There are some enzymes which have relatively low specificities on substrates but have high bond specificity. They will catalyse a wide range of substrates provided they contain the required chemical bond. However, many enzymes show absolute or near absolute specificity where they will only catalyse reactions with a single specific substrate at an appreciable rate. Another distinct feature of many enzyme catalysed reactions is their stereospecificity.

(iii) The enzymatic activity may be regulated by small ions or other molecules.
The unique catalytic abilities of enzymes and the possibility for the reaction to be carried out under mild conditions have long attracted great interests of scientists to attempt to utilize enzymes for industrial applications. In the 1960's,

the first successful application of enzyme for industrial production was achieved by Chibata and co-workers(Izumi, Chibata and Itoh, 1978) in Japan. The success in the application of enzyme in food industries is examplified by the large scale production of high fructose syrup from enzymatically hydrolysed starch by means of glucose isomerase. These successes led to the development of a new technological discipline: Enzyme Technology. Today, enzyme technology can be considered as one of the most important areas of biotechnology, the essence of which is to produce products via biotransformation. Thus one may claim that enzymes play a pivotal role in biotechnology.

Enzymes are used in preference to other catalysts in many industrial and biological processes. This is because these enzymatic processes are more efficient by minimizing the production of undesirable byproducts and thus increase plant capacities per unit cost. In addition, less energy is required because enzyme reactions can be carried out at low temperatures.

1.2 Our research activities

Our research activities include three aspects of enzyme technology.

(i) The isolation and characterization of enzymes from natural sources especially from food and agricultural sources and the elucidation of the functional roles of enzymes in relation to the quality of these products is of fundamental importance in enzyme technology. The objective of our project on the studies of Malaysia cocoa beans polyphenoloxidase and proteases is to attempt to delineate the involvement of enzymatic processes in relation to the quality index in Malaysia cocoa beans and hence to find ways to control these processes and to improve the quality of the Malaysia cocoa beans. In these studies we have successfully isolated the cocoa beans enzymes and their characteristics in aqueous solution have been studied (Lee, *et al.*, 1990-1991).

(ii) Studies of enzymatic reactions in non-aqueous solvent system is another active area of research in enzyme technology. The objective of our project on the studies of elastase and thermolysin is to probe the catalytic reaction at the molecular level, to sort out the optimal reaction conditions to develop industrially viable systems to synthesise the dipeptide sweetener, aspartame, which is 180 times sweeter than sucrose. A new approach was developed for the synthesis of this industrially important sweetener precursor (Lee, *et al.*, 1992).

(iii) Immobilization of enzyme to insoluble supports is also a topic of interest in enzyme technology. Enzymes are water soluble and are sensitive to pH of the solution, heat, organic solvents and heavy metals. They can easily be denatured by these environmental factors. Consequently, one of the main research areas of enzyme technology is to devise methods to stabilize enzymes to withstand

harsh conditions encountered during isolation or purification and industrial processing. The cost effectiveness of an enzymatic process is also the main concern of enzyme technology. If the immobilization of enzyme to insoluble supports produce an immobilized biocatalyst with high retention of catalytic activity and high operational stability, enzymes can be easily recovered and reuse. The objective of our project on the studies on immobilization of aminoacylase is to develop useful immobilized systems for the production of *L*-phenylalanine.

L-phenylalanine is one of the 10 essential amino acids which can be given to patients by intravenous infusion. In addition it is used to produce aspartame. It may also be used to improve the quality and flavour of food.

In this paper, only the work of aminoacylase is presented.

1.3 Immobilization of aminoacylase

Aminoacylase catalyses the optical resolution process of racemic mixtures of *N*-acetyl-*DL*-phenylalanine according to the reaction given below:

$$\textit{N}\text{-acetyl-}\textit{DL}\text{-phenylalanine} + H_2O \xrightarrow{\text{aminoacylase}} \textit{L}\text{-phenylalanine} + \textit{N}\text{-acetyl-}\textit{D}\text{-phenylalanine}$$

(which can be recemised)

Aminoacylase selectively hydrolyses the *N*-acetyl-*L*-isomer. The liberated amino acid can then be separated from the unhydrolysed *N*-acetyl-*D*-isomer based on their different solubilities in the solution.

There are four methods for enzyme immobilization:

(1) Physical absorption. The solid support has an affinity for the enzyme and the enzyme is absorbed on the surfaces of solid supports.

(2) Binding to solid matrixes. The enzyme is bound to the water insoluble support by ionic or covalent bonds.

(3) Crosslinking. Using bi- or multifunctional crosslinking reagents to crosslink the enzymes into large insoluble aggregates.

(4) Entrapping. There are two ways of entrapping: a) lattice type entrapping method. This involves entrapping the enzyme with the interstitial spaces of a crosslinked water insoluble polymer and (b) microcapsule type: enclosing enzyme in a semipermeable polymer membrane.

In this work, we immobilize aminoacylase by microencapsulation with calcium alginate . Microencapsulation is generally a very mild immobilization technique

for biomacromolecules, microorganisms or organelles (Mattiasson, 1983). Sodium alginate is a readily available and non-toxic polysaccharide that is a suitable material for immobilization of biomolecules and cells by entrapment (Mattiasson, 1983). In fact, alginate has been used quite extensively for microencapsulating or entrapping cells or microorganisms for biotransformation. On the other hand, its use for immobilizing enzymes or proteins remains rather limited. This is primarily due to the fact the pores of the alginate beads are too large to be useful for protein molecules and result in serious leakage problems. Consequently, if means are to be found to alleviate such problems the scope of utilizing calcium alginate beads as an immobilized matrix for enzymes and proteins would be greatly expanded. Therefore we have investigated methods to stabilize the calcium alginate beads. To accomplish this we coated the calcium alginate beads with various reagents such as: polyethyleneimine, polyethyleneimine-glutaraldehyde, poly-L-lysine and covalent crosslinking of enzyme to the beads.

2. MATERIAL AND METHODS

2.1 Enzyme and chemical reagents

All materials used were described by Lee et al (17, 19).

2.2 Preparation of immobilized aminoacylase

2.2.1 Entrapment in calcium alginate beads

Ten cm^3 aminoacylase solution (50 mg cm^{-3}) was added to 10 cm^3 of 4% (w/v) sodium alginate solution.The mixture was stirred for 10 min and allowed to equilibrate for a further 10 min. The mixture was then added dropwise into a gently stirred 150 cm^3 0.2 mol dm^{-3} $CaCl_2$ solution. Pear shape transparent beads (2.8 x 3.1 mm in diameter) were allowed to harden in the $CaCl_2$ solution for another 30 min. They were filtered by suction with a sintered glass funnel, washed thoroughly with distilled water, air-dried and stored at 4 °C until use.

2.2.2. Entrapment in calcium alginate beads coated with PLL (PLL-coated calcium alginate beads)

Preformed calcium alginate beads (10.0 g) entrapping with enzyme prepared as described above were suspended in 200 cm^3 of 0.25% PLL solution (pH 7.0) for 10 min with stirring. The beads were then washed with distilled water, air dried and kept at 4 °C until use. The size of the bead was similar to the uncoated beads.

Activity assay, stability test and methods of characterization were performed as described by Lee et al (17-21).

3. RESULTS AND DISCUSSION

Immobilization of aminoacylase in stabilized calcium alginate beads

The properties of various immobilized systems were summarised in Table I. It was noted that the optimum pH, optimum temperature and the thermal stability in the immobilized systems studied do not change greatly from the free enzyme. This seems to suggest that the conformation of enzymes do not change greatly after immobilization. The possible reason for the slightly larger values of Km and Vmax for the immobilized calcium alginate beads were discussed (Lee, *et al.*, 1992, 1993). The present studies showed that encapsulating aminoacylase in PLL-coated calcium alginate beads provided an efficient system of enzyme immobilization. The resulting capsules not only prevented enzyme leakage but also retained high biological activity. It is possible that the coating is a surface phenomenon, therefore, PLL did not deactivate the enzyme entrapped in the interior of the calcium alginate beads. This immobilized system is further illustrated in this paper.

Table I The Enzymatic Properties of Free and Immobilized Aminoacylase

Properties	Free Aminoacylase	Cag	Pei-Coated Cag	PLL-Coated Cag	Partially Purified Aminoacylase	Cross-Linked Cag
Km (mol dm^{-3})	0.88	14.29	3.03	11.11	1.95	3.33
Vmax (μmole/min)	0.021	0.695	0.024	0.228	0.034	0.12
Optimum pH	6.0 - 7.0	6.0 - 6.4	6.0	6.5	6.5 - 7.2	6.4
Optimum Temperature (°C)	60	65	60	55	60	55
Activation energy (Kj/mol)	16.7	15.2	31.3	24.2	16.8	27.3
Heat stability (°C)	62	58	58	58	54	50
Specific activity (μmole/min/mg)	1.99	5.90	0.69	5.50	19.10	2.22
Operational stability half-life (cycle)	-	2	>10	>10	-	4

**50% of the enzyme activity was lost after heat treatment at the specified temperature for 10 min.*

3.1 Surface structure of PLL coated calcium alginate beads

It seems that ionic interactions between the positively charged amino groups and the functional groups of alginate molecules are responsible for the stabilization of the beads. The ionic interactions resulted in the formation of a network structure on the surface of calcium alginate beads as revealed by the scanning electron micrographs (Fig. 1 A). This structure prevents enzyme leakage. The surface structures of the beads

were identical to those stabilized calcium alginate beads reported earlier but different from the uncoated calcium alginate beads (Fig. I B) This preparation alleviates the enzyme from leaking.

It was estimated that about 30 % of enzyme was encapsulated in the beads and the specific activity of immobilized aminoacylase was 5.4 μmol min^{-1} mg^{-1}.

3.2 Characterization of PLL-stabilized calcium alginate beads

3 2.1. Thermal stability

The thermal stability profiles for the immobilized aminoacylase entrapped in uncoated and PLL-coated calcium alginate beads were given in Fig. 2. The activity decreased gradually as the temperature of incubation was increased from 30–50 °C. On the contrary, the enzyme in the uncoated beads was stable in this temperature range. In addition, when the temperature of incubation was increased from 58 to 60 °C, the enzyme activity dropped drastically. About 50 % and 30% drop in activity were observed for the immobilized enzyme in PLL-coated and uncoated beads respectively. This suggests that the immobilized aminoacylase in PLL-coated calcium alginate beads is more susceptible to thermal deactivation. It is possible that the heating at 58 °C induces conformational changes of PLL which may lead to the loosening of the network structure resulting in enzyme leakage. Further work is in progress to unravel this.

3.2.2. Optimum temperature

The optimum temperature profile is given in Fig. 3. The optimum temperature for the immobilized aminoacylase encapsulated in PLL-coated calcium alginate beads was 55 °C, which was about 10 °C lower than that in the

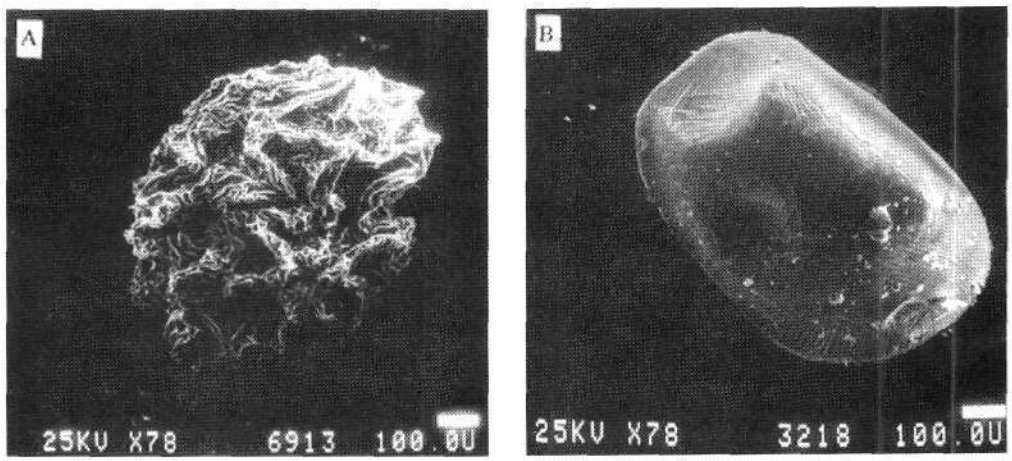

Fig. 1 **Scanning electron micrographs of (A) PLL-coated calcium alginate beads and (B) uncoated calcium alginate beads. The scanning electron micrograph was made as described by Lee *et al.*[6]**

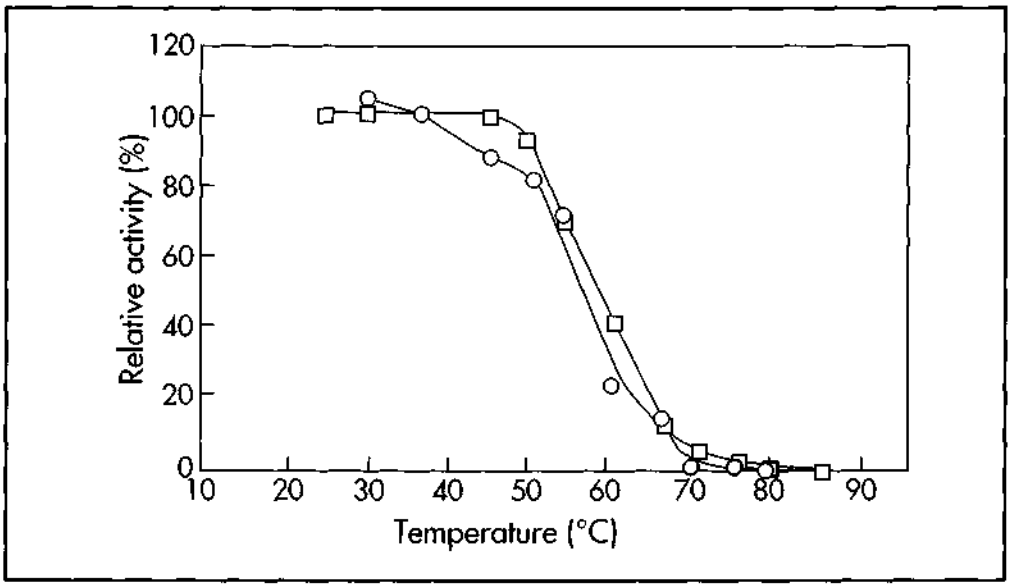

Fig. 2 Effect of temperature on the stability of aminoacylase. A mixture of 0.6 cm^3 of 0.5 mmol dm^{-3} $CoCl_2$ and 60 mg of calcium alginate beads entrapped with enzyme was incubated at various temperatures for 10 min. It was then rapidly cooled in an ice bath. The residual activity was then assayed by the standard method. The determined residual activities at various temperatures were calculated with respect to that at 37°C. Uncoated calcium alginate beads (□), PLL-coated calcium alginate beads (○).

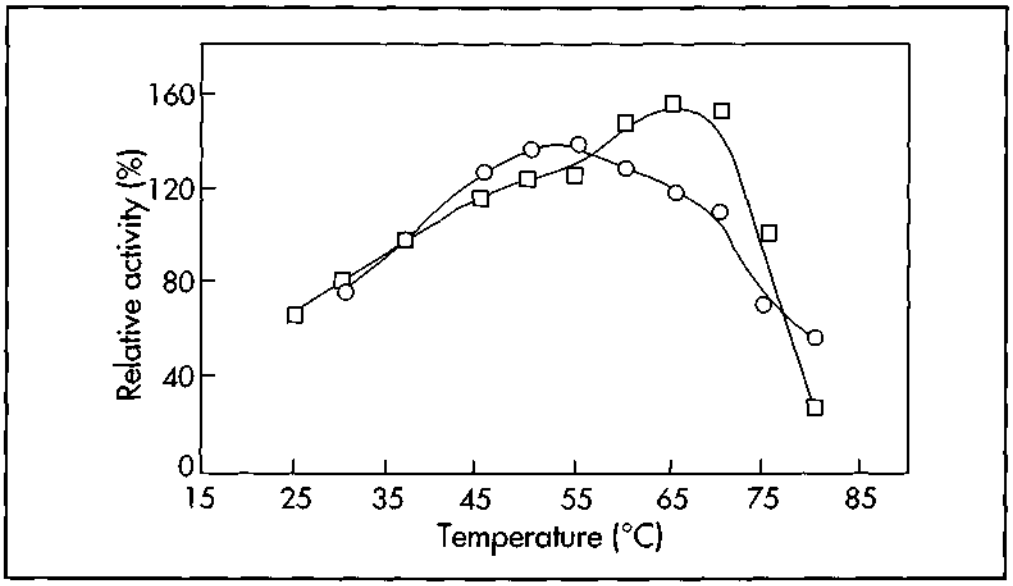

Fig. 3 Optimum temperature studies of immobilized amino-acylase. The enzyme activities at various temperatures were measured by the standard method. They were then calculated with respect to that at 37°C. Uncoated calcium alginate beads (□), PLL-coated calcium alginate bead (○).

uncoated beads. These results are consistent with our earlier suggestion that PLL may have undergone conformational changes which lead to enzyme leakage. The activation energy determined from the Arrhenius plot was 24.2 kJ/mol, greater than those for the free enzyme and the uncoated beads . The higher activation energy observed will be explained later.

It is interesting to note that the optimum temperature for the enzyme activity was at 55 °C but about 50 % of the activity was lost at about 58 °C after 10 min incubation at this temperature. These observations may arise from the stabilization of the enzyme by the formation of enzyme-substrate complex in the optimum temperature studies.

3.2.3 Effect of pH

The pH profiles of the immobilized enzyme in uncoated and PLL-coated beads were identical(data not shown). The results indicated that the coating of calcium alginate beads with PLL probably exerted minimal perturbation on the polarity of the microenvironment of the encapsulated enzyme. As a result, the microenvironment was similar to that prevailed in the free enzyme.

3.2.4 Kinetic constants

The values of K_m amd V_{max} determined for the immobilized enzyme in PLL coated beads from a Lineweaver-Burk plot (Fig. 4.) were 11.11 mmol dm^{-3} and 0.076 μmol min^{-1} respectively and for those in uncoated beads were 14.29 mmol dm-3 and 0.695 μmol min^{-1} respectively. These results lend support to our suggestions, (IT) the uncoated beads provide a higher concentration of substrates in close proximity to the enzyme and the possible interaction of the carboxylate ions of the substrate with the amino groups of PLL retard the diffusion of substrates and leads to the lower K_m and V_{max} values for this immobilized system when compared with the uncoated beads. This also explained the higher activation energy observed for this immobilized system.

3.3. *Operational stability*

3.3.1 Batch operation

The significant feature about the PLL coated calcium alginate beads was its high operational stability. Fig. 5 shows the operational stability of immobilized aminoacylase. The immobilized enzyme was stable after repeated batch operations. Only about 5% decline of activity was detected after ten reaction cycles and the activity profile of the operation remained steady from the fourth reaction cycle onwards. The loss of activity for the first three reaction cycles was probably due to the desorption of enzyme that was attached loosely on the surface of the beads during the operation. However, the encapsulated enzyme was stable and remained active. Inferring from the activity profile of the batch operation of the immobilized enzyme, the performance of this system can be projected to persist for

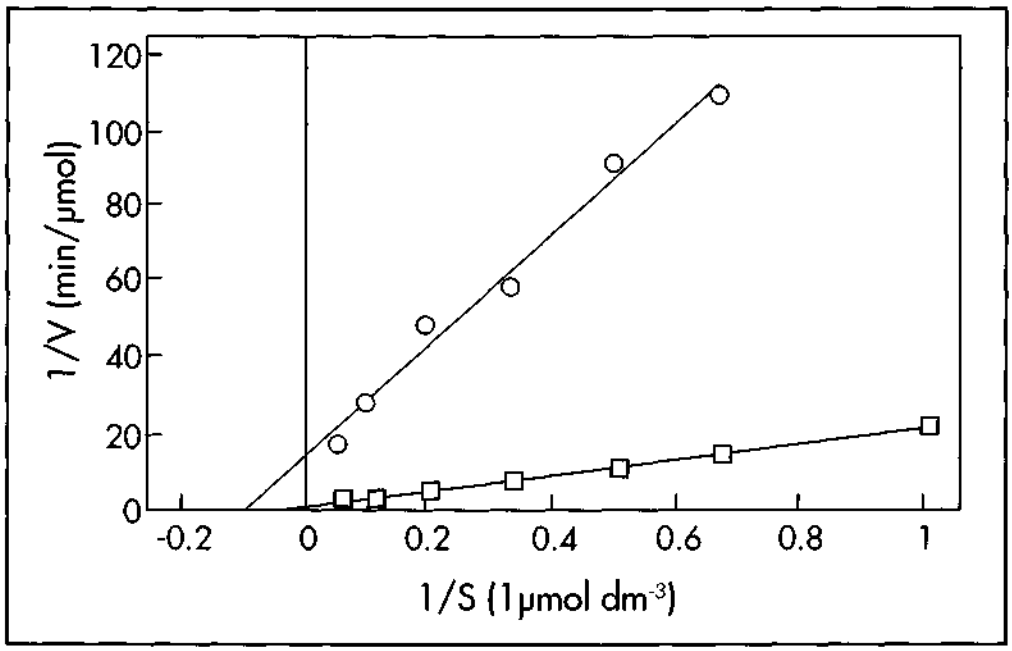

Fig. 4 Lineweaver-Burk plot of immobilized aminoacylase in uncoated (□) and PLL-coated calcium algiante beads (○).

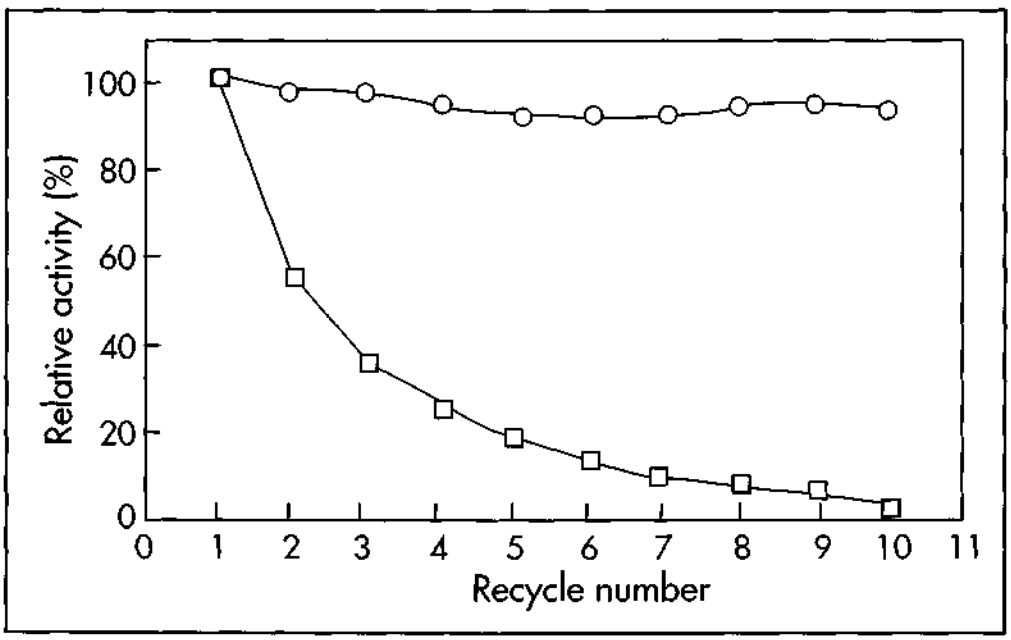

Fig. 5 Operational stability of uncoated calcium alginate beads (□) and PLL-coated calcium alginate beads (○) entrapped with aminoacylase. 100 mg of PLL-coated calcium alginate beads was used to determine the activity under standard conditions. The beads were then rinsed with distilled water and assayed for the second cycle and so forth.

many cycles. These results showed that PLL did not introduce instability or denaturing effects on the enzymes as mentioned earlier.

In addition, studies on the storage stability showed that PLL-stabilized calcium alginate beads were stable for at least 50 days on storage at 4 °C. During this period, no significant loss of enzyme activity was detected. The high storage and operational activities of this system may be due to the proteineous and hydrophilic nature of PLL which is biocompatible with the enzyme molecule.

3.3.2 Bioreactor continuous operation

The PLL-stabilized calcium alginate beads entrapped with immobilized aminoacylase were packed into a bioreactor for continuous operation performance assay. Fig. 6 shows the yield of the product produced by the bioreactor at various time intervals. When a solution mixture containing 20 mmol dm^{-3} *N*-acetyl-*DL*-phenylalanimne and 10 mmol dm^{-3} $CaCl_2$ and 0.1 mmmol dm^{-3} $CoCl_2$ passed through the bioreactor with a flow rate of 2.7 cm^3 min-1, it was found that the yield of the product increased with time and reached an equilibrium after about 16 h with product yield of about 68 % (Fig. 6 (a)). The system was very stable and no further loss in activity was detected for a period of 600 h. of continuous operation (Fig. 6 (b)). When the initial substrate concentration was reduced by half to 10 mmol dm^{-3} of *N*-acetyl-*DL*-phenylalanine, the yield reached about 80% accompanied by a further rise to the steady state (data not shown).

If a faster and quantitative conversion of the substrate is desired, one may reduce the substrate loading or increase the amount of enzyme loading in the PLL-stabilized calcium alginate beads or increase the packing dimension of the bioreactor.

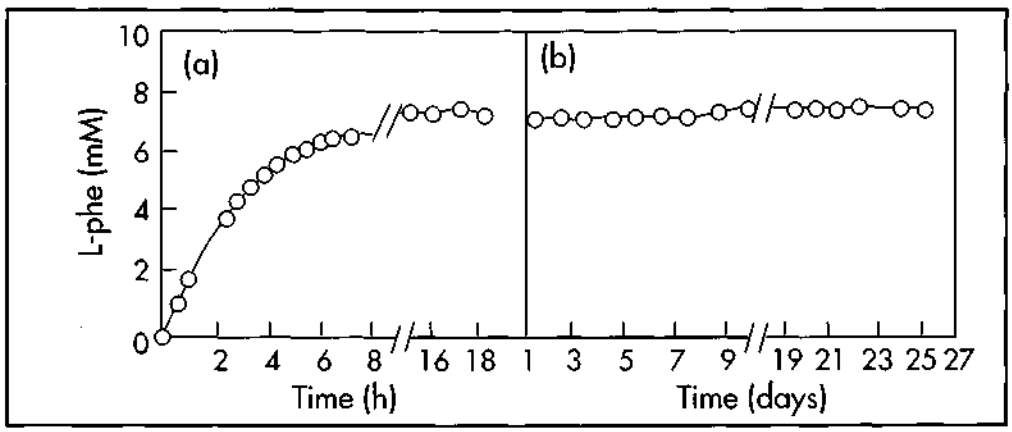

Fig. 6 Continuous operatin of PLL-coated calcium alginate beads entrapped with aminoacylase in a bioreactor. 3 g beads were packed into a column (20 cm x 1.5 cm) with a bed volume of 7.5 cm^3. A solution mixture of 20 mmol dm^{-3} *N*-acetyl-DL-phenylalanine, 10 mmol dm^{-3} $CoC1_2$ and 0.1 mmol dm^{-3} $CoC1_2$ was charged into the column with a flow rate of 2.7 $cm^3 h^{-1}$ at room temperature.

4. CONCLUSION

The present studies showed that encapsulating aminoacylase in PLL-coated calcium alginate beads provided an efficient system of enzyme immobilization. Hitherto it was the best system of various stabilized calcium alginate beads studied for the production of L-phenylalanine. The resulting capsules not only prevented enzyme leakage but also retained a high biological activity. As the coating is a surface phenomenon, PLL did not deactivate the enzyme entrapped in the interior of the calcium alginate beads.

The operational and storage stabilities of the PLL-stabilized beads were excellent. No appreciable decrease in activity was detected after ten reaction cycles and when stored at 4 °C for 1200 h. A plug and flow type of bioreactor was constructed for the immobilized enzyme. When the bioreactor was operated under different substrate concentrations, there was no detectable change in activity after equilibrium has been reached for at least 25 days. Under the operational conditions used, a high conversion yield of 80% was achieved readily. The results of the present studies showed that the scope for the utilization of calcium alginate beads as a convenient immobilized matrix for enzyme and other biological molecules and microorganisms can be expanded by reacting ionically with PLL to form a membrane network on the surface of the calcium alginate beads.

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